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Combining Suppression Subtractive Hybridization and Microarrays To Map the Intraspecies Phylogeny of *Flavobacterium psychrophilum*

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Reciprocal subtractive libraries were prepared for two strains of *Flavobacterium psychrophilum*, one virulent and the other avirulent in a trout challenge model. Unique clones were sequenced and their distribution assessed among 34 strains. The analysis showed that *F. psychrophilum* is composed of two genetic lineages, possibly reflecting host specificity.

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease and rainbow trout fry syndrome, both of which affect salmonid fish and impact commercial aquaculture and resource enhancement hatcheries worldwide (12, 19, 21). Coho salmon (*Oncorhynchus kisutch* [Walbaum]) and rainbow trout (*O. mykiss*) are particularly susceptible, although *F. psychrophilum* also infects other fish species (21). *F. psychrophilum* strains vary greatly in the ability to establish disease (virulence). For example, one well-studied strain (ATCC 49418 [4, 6]) is unable to cause significant mortality (9) while strain CSF 259-93 causes high mortality (16) in a trout challenge model. There are no commercial vaccines available for bacterial coldwater disease, although several research groups have active programs in this area (11, 14, 20). From these studies we know that not all strains elicit an antibody response that is effective against other strains and consequently there may be considerable genetic variation between strains.

The goals of this project were (i) to examine genetic differences between two strains of *F. psychrophilum* (CSF 259-93 [9] and ATCC 49418 [16]) and (ii) to assess the extent and distribution of genetic variation between other strains relative to geographical source and host species. Strains for the latter assessment were chosen based on availability at the time of this study. All strains were stored at −80°C and were cultured at 16 to 17°C in tryptone yeast extract salts medium (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2). Genomic DNA (gDNA) was extracted using the DNeasy tissue kit (QIAGEN, Valencia, CA).

We prepared reciprocal suppression subtractive hybridization libraries for the two strains by using the PCR-Select Bacterial Genomic Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol, except that tester and driver gDNAs were restriction enzyme digested with RsaI (supplied with the kit) and DraI (New England Biolabs, Inc,, Beverly, MA) and the hybridization step was at 59°C. Resulting DNA fragments were cloned in pCR2.1 (Invitrogen, Carlsbad, CA), and 576 randomly chosen recombinant clones were used to make a microarray as previously described (8, 10). gDNA (0.5 μg) was nick translated for 2 h in the presence of biotin-dATP (BioNick Labeling System; Invitrogen) and hybridized to the microarray (data not shown). Microarray slides were processed, imaged, and analyzed as described previously (23). Using stringent selection criteria, where the median probe intensity was ≥95% of the maximum pixel intensity in one strain and ≤5% of the maximum pixel intensity in the other strain, we identified 130 (23% of the total) unique clones that were retrieved from the library and sequenced. Successfully sequenced clones (n = 124) were queried using the National Center for Biotechnology Information BLASTx server (1), and each was assigned a general function based on the significantly similar proteins (e score, $\leq e^{-5}$). Twenty-one of the clones were redundant (Table 1). Sixteen of the remaining 103 clones (15%) were confirmed to be unique to the relevant strain by using PCR (data not shown). DNA fragments unique to CSF 259-93 included four classes of proteins. Notably, a large percentage (13% of the total) of the sequences encoded restriction-methylation systems that differ between the two strains, and this may explain the difficulties encountered by other researchers attempting to transform plasmid DNA into *F. psychrophilum* strains (2). We identified five putative virulence genes that were exclusive to *F. psychrophilum* CSF 259-93 by both microarray hybridization and PCR (Table 2).

The two *F. psychrophilum* strains also were analyzed for

TABLE 1. Numbers and groups of unique clones found in *F. psychrophilum* strains ATCC 49418 and CSF 259-93

Protein family	Total no. of clones (no. of redundant clones)	
	ATCC 49418	CSF 259-93
Replicase A protein	10 (1)	11 (7)
DNA/RNA digestion or modification	12 (0)	15 (3)
Enzymes in biochemical pathways	3 (0)	4 (0)
Unknown and hypothetical proteins	30 (2)	29 (6)
Major facilitator superfamily transporters	0	4 (2)
ATP-binding cassette transporters	0	2 (0)
Outer membrane (integral and membrane-associated) proteins	0	3 (0)
Pathogenesis-related proteins	0	1
Total	55 (3)	69 (18)

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TABLE 2. Sequence analysis of *F. psychrophilum* CSF 259-93-specific virulence candidate genes[a]

SSH clone	Insert size (bp)	BLASTx E score	Similar protein (accession no.), organism	Predicted function or property
csf1-d7	337	$8e^{-36}$	BspA (AF054892), *Bacteroides forsythus*	Cell surface antigen
csf2-c4	996	$1e^{-14}$	Chut2700 (ZP_00119301), *Cytophaga hutchinsonii*	Outer membrane component of type II secretory pathway, component PulD
csf2-g12	346	$2e^{-39}$	PP2193 (AAN67806), *Pseudomonas putida* KT2440	Putative outer membrane ferric siderophore receptor
csf3-c2	518	$2e^{-10}$	RL009 (AAP84136), *P. aeruginosa* PA14	Putative pathogenesis-related protein (contains RGD motif)
csf1-c10[b]	677	$8e^{-48}$	RT1B_ACTPL (P26760), *A. pleuropneumoniae*	Toxin RTX-I secretion ATP-binding protein
csf3-c12[b]	492	$4e^{-08}$	RT1B_ACTPL (P26760), *Actinobacillus pleuropneumoniae*	Toxin RTX-I secretion ATP-binding protein

[a] GenBank accession numbers are AY823250 to AY823255.
[b] Two clones align with the same protein at different positions.

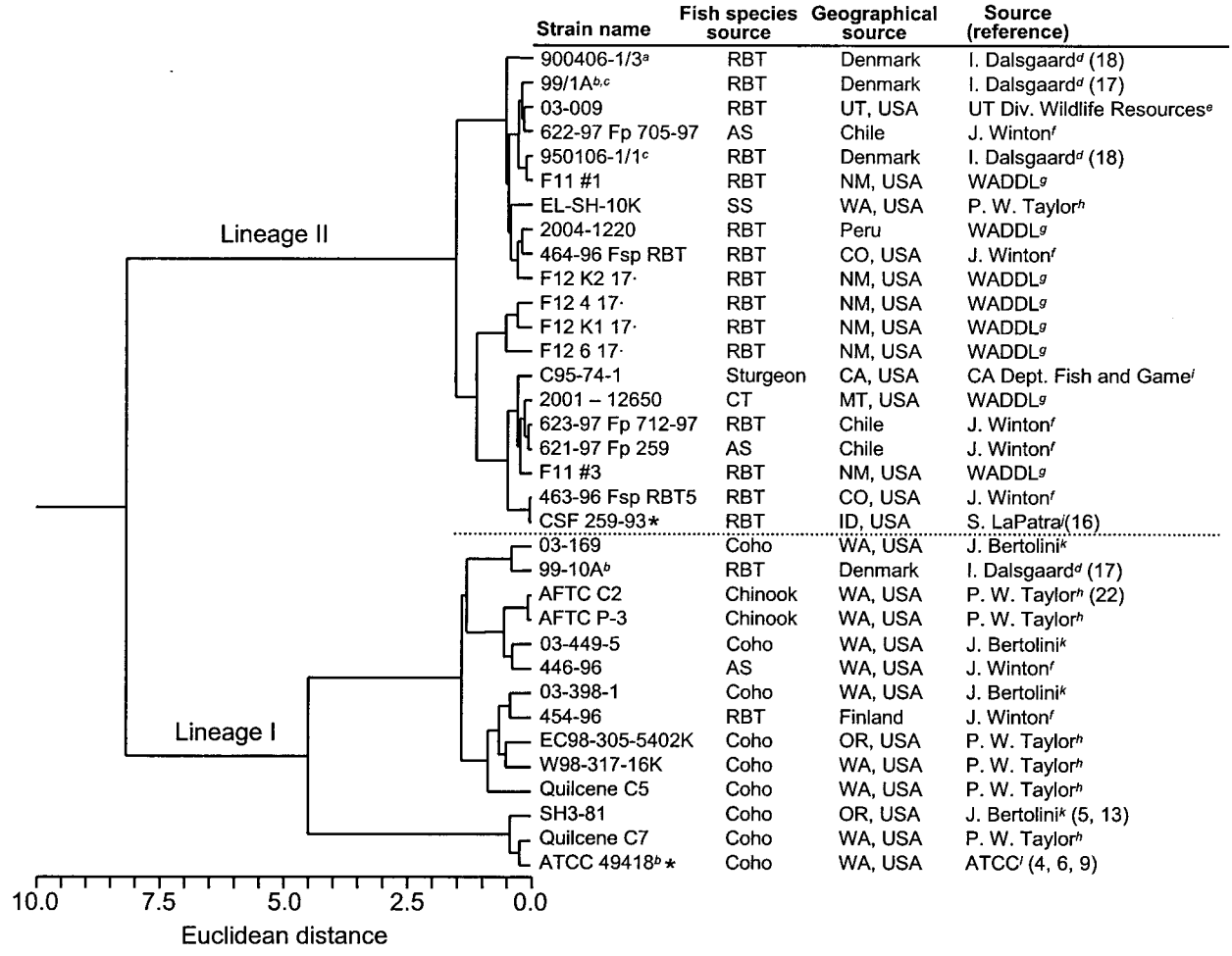

FIG. 1. Cluster analysis of 34 *F. psychrophilum* strains based on microarray hybridization pattern for 103 probes identified from reciprocal subtractive hybridization experiments. RBT, rainbow trout (*O. mykiss*); AS, Atlantic salmon (*Salmo salar*); SS, steelhead salmon (*O. mykiss*); sturgeon (*Acipenser transmontanus*); CT, cutthroat trout (*O. clarki*); Coho, coho salmon (*O. kisutch*); Chinook, chinook salmon (*O. tshawytscha*). Superscripts: *a*, serotype Th; *b*, serotype Fp^T; *c*, serotype Fd (18); *d*, Ministry of Food, Agriculture and Fisheries, Denmark; *e*, Salt Lake City, Utah; *f*, U.S. Geological Survey Western Fisheries Research Center, Seattle, WA; *g*, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman; *h*, Abernathy Fish Technology Center, Longview, WA; *i*, Sacramento, CA; *j*, Clear Springs Foods, Inc., Buhl, ID; *k*, Northwest Indian Fisheries Commission, Olympia, WA; *l*, American Type Culture Collection, Manassas, VA.

biochemical, phenotypic, and 16S rRNA gene differences. ATCC 49418 hydrolyzed gelatin, a common property of *F. psychrophilum* strains that is thought to be correlated with virulence (5), but the absence of gelatin hydrolysis by the virulent strain suggests it is not necessary for virulence in the trout challenge model. CSF 259-93 was able to grow slowly at 30°C, which is unusual for *F. psychrophilum* (21), while ATCC 49418 was unable to grow at this temperature. ATCC 49418 was susceptible to tetracycline in a Kirby-Bauer disk diffusion test (3) (zone size, 42 mm), while CSF 259-93 showed significant resistance (zone size, 16 mm), which supports our finding that one of the sequences unique to CSF 259-93 encodes a putative TetA protein. Although both strains were positive for an *F. psychrophilum*-specific PCR assay (22), when the entire 16S rRNA gene sequences were compared they differed by six nucleotides (two triplets) within a 12-nucleotide region of the gene (GenBank accession numbers AY662493 and AY662494).

The distribution of the 103 unique sequences (resulting from the reciprocal suppression subtractive hybridization experiments) among 34 *F. psychrophilum* isolates was analyzed (Fig. 1). The identity of all isolates was verified by using a microarray containing 16S rRNA gene probes from 15 known fish pathogens (23) with the addition of a second *F. psychrophilum* 16S rRNA gene probe that was unique to the CSF 259-93 strain (M. Soule et al., unpublished data). All 34 of the isolates were confirmed to be *F. psychrophilum* by this method. A subset of 11 isolates was confirmed as *F. psychrophilum* using nested PCR (22). Biotin-labeled gDNA from each of the 34 isolates was hybridized to the subtractive library microarray, and data from 103 unique probes were used to construct a dendrogram (Ward's minimum-variance cluster algorithm; NCSS, Kaysville, UT). In this analysis, strains that shared more probe sequences were considered more genetically related compared to strains that shared fewer probe sequences. Presence or absence of gDNA was determined using a previously published algorithm (7). Only seven strains were positive for all five putative virulence genes (data not shown). Three of the isolates used in this analysis were known to be highly virulent in a trout challenge model: CSF 259-93, SH3-81, and 950106-1/1 (13, 15, 18). Only virulence gene candidate csf1-d7 (BspA like) is present in all three of these isolates and thus may be a target for vaccine development and studies of pathogenic mechanisms.

The dendrogram (Fig. 1) was largely dichotomous, with 13 strains grouping with ATCC 49418 (lineage I) and 19 strains grouping with CSF 259-93 (lineage II). Euclidian distance between these two lineages was similar to what can be expected for lineage differences that have been documented for *Listeria monocytogenes* (8, 10). Both lineages included strains isolated from Europe and North America, while lineage II included isolates from Chile and Peru. All but two of the strains in lineage I originated from salmon (coho, chinook, and a single strain, 446-96, from Atlantic salmon) from the Pacific Northwest (Washington and Oregon). The two remaining strains (454-96 and 99-10A) in lineage I were from rainbow trout in Europe. Conversely, only one strain (EL-SH-10K) in lineage II originated from Washington or Oregon and none originated from chinook or coho salmon (two strains originated from Atlantic salmon). Almost all of the strains in lineage II are from rainbow trout. Thus, there is clear genetic separation into at least two major lineages and this finding is most strongly associated with the host species and less strongly associated with geographic origin.

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